The marriage of quantitative genetics and cell biology

A novel screening approach reveals people have genetically encoded variation in microtubule stability

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Abbreviations: SNP, single nucleotide polymorphism; TUBB, tubulin beta; TUBA, tubulin alpha; GWAS, genome-wide association study; eQTL, expression quantitative trait loci; LCLs, lymphoblastoid cell lines; Hi-HOST, high-throughput human in vitro susceptibility testing; APIP, apaf-1 interacting protein

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Microtubules play a central role in many essential cellular processes, including chromosome segregation, intracellular transport, and cell polarity. As these dynamic polymers are crucial components of eukaryotic cellular architecture, we were surprised by our recent discovery that a common human genetic difference leads to variation in microtubule stability in cells from different people. A single nucleotide polymorphism (SNP) near the *TUBB6* gene, encoding class V β -tubulin, is associated with the expression level of this protein, which reduces microtubule stability at higher levels of expression. We discuss the novel cellular GWAS (genome-wide association study) platform that led to this discovery of natural, common variation in microtubule stability and the implications this finding may have for human health and disease, including cancer and neurological disorders. Furthermore, our generalizable approach provides a gateway for cell biologists to help interpret the functional consequences of human genetic variation.

Expanding the Cell Biologist's Toolbox to Cellular GWAS

Cell biology has long been a multi-disciplinary endeavor with an ever-expanding set of tools to increase our resolution and understanding of cellular processes. One of the cell biologist's main tools has been genetics: to deplete, knockout, or

overexpress proteins of interest (reverse genetics), to perform cellular screens of function (forward genetics), to manipulate genes by adding tags for protein localization and purification studies (genetic engineering), and to examine the genome-wide consequences of cellular perturbations (genomics). In addition to these molecular genetic-based approaches, cell biologists have also used naturally occurring genetic variation to study biology as well, primarily by studying cells derived from patients with specific Mendelian disorders; for example, cells with *CFTR* mutations from cystic fibrosis patients. A less common approach is to use the common genetic diversity among healthy people to study cell biology. However, by phenotyping genotyped cells for cell biological traits, the same genome-wide association approaches that have become common for identifying risk alleles for complex diseases,^{1,2} can be used to decipher how genetic differences contribute to variation in cellular traits among people. In this regard, most of the attention thus far has been focused on genome-wide association of gene expression levels. Several labs have identified expression quantitative trait loci (eQTLs), genetic differences that are correlated with the expression level of genes.^{3–6} Many of these studies take advantage of lymphoblastoid cell lines (LCLs; Epstein Barr virus-immortalized B cells) that have already been genotyped by the HapMap^{7,8} and 1000 Genomes⁹ projects. Using these cells, researchers have measured gene expression using microarrays or RNA-seq

and for each transcript determined how well each of millions of SNPs can explain the observed phenotypic variation. The SNPs that show strong association may include, for example, SNPs that change transcription factor binding or mRNA stability. In this way, researchers have assembled lists of human genetic differences that affect cellular readouts, in this case, gene expression.

Our lab applies this basic idea of GWAS of cellular traits to host-pathogen phenotypes through a platform called Hi-HOST (High-throughput human in vitro susceptibility testing).^{10,11} Hundreds of LCLs are infected with identical doses of a single pathogen, and we measure multiple complex cellular traits including invasion, cell death, and cytokine response. Importantly, because the genetic differences are associated with cellular traits, findings are accessible to functional testing by the other approaches in the cell biologist's toolbox. We have been applying this approach to multiple pathogens, with the greatest focus on *Salmonellae*. Pathogens act as bioarchitects of the cell, manipulating structures and signaling pathways to facilitate their ability to thrive in hosts. For example, *Salmonella* invades host cells by inducing a form of endocytosis called macropinocytosis, allowing us to use it as a biological probe for elucidating the mechanisms of macropinocytosis.

Previously, we identified and characterized a common genetic variant associated with both pyroptosis, a caspase-1-dependent pro-inflammatory form of cell death induced by pathogens including *Salmonellae*, and risk of bacteremia and death in sepsis patients.¹² This SNP was also associated with altered expression of the *APIP* (*apaf-1 interacting protein*) gene. Unexpectedly, the mechanism for this effect appeared to occur through APIP's previously unexplored role as a methionine salvage enzyme,¹² which has since been confirmed by two other labs.^{13,14} This example demonstrates how cellular GWAS of a complex trait (pyroptosis) can lead to unexpected connections among cell biological pathways and also connections to risk and outcomes of related diseases.

Cellular GWAS and Follow-Up Reveals Humans Vary in Microtubule Stability

The SNP near *APIP* was just one eQTL associated with the pyroptosis phenotype, and characterizing other SNPs associated with this and other pathogen-induced phenotypes will reveal additional insights into cell biology and disease. Recently, we characterized a SNP identified in the same Hi-HOST cellular GWAS for pyroptosis that was associated with the expression of *TUBB6* (tubulin, β 6 class V).¹⁵ *TUBB6* had been previously described as a novel tubulin isoform because upon overexpression it results in a complete dismantling of the microtubule network.¹⁶ We found that cells with the SNP allele that was correlated with decreased *TUBB6* expression demonstrated increased microtubule stability. An inverse relationship between *TUBB6* expression and microtubule stability was verified in both LCLs and primary fibroblasts from different people, as assessed both by tubulin acetylation and flow cytometric measurement of polymerized tubulin. Increased microtubule stability, either naturally by having the SNP or experimentally through *TUBB6* RNAi or the drug paclitaxel (Taxol), increased *Salmonella*-induced pyroptosis. Overexpressing *TUBB6* via transfection had the anticipated reverse effect and inhibited caspase-1 dependent cell death. This indicates that *TUBB6* is an inhibitor of pyroptosis, and thus that pyroptosis is regulated by microtubule stability. We hypothesize that microtubule stabilization may make the plasma membrane more rigid and thus more prone to lysis, while less stable microtubules leave the plasma membrane more flexible and adaptable to cellular swelling. However, microtubules may have multiple roles in caspase-1 activation and the pyroptotic response (as exemplified by microtubule disruption by colchicine unexpectedly *increasing* pyroptosis), and further research is necessary to characterize these mechanisms.

To our knowledge, the SNP near *TUBB6* is the first example of common germline genetic variation affecting microtubule stability, but others have identified genetic variation in microtubule structure that is functionally important.

Somatic mutations in tubulin genes in tumors often contribute to resistance to cancer drugs, such as paclitaxel. Yin et al. characterized three SNPs in the class I β tubulin gene (*TUBB1*) that increased resistance to paclitaxel in cell culture.¹⁷ One of these SNPs was found in breast cancer and two in hematologic malignancies.^{18,19} These resulted in amino acid changes in important protein domains, causing disrupted microtubule networks and decreased microtubule content. Mutations in class III β tubulin (*TUBB3*) resulting in tumoral overexpression have been associated with resistance to many microtubule-inhibiting drugs.^{20,21} Mutations in the drug-binding region can also arise and result in resistance, although these are less common.^{20,22}

Possible Implications of Microtubule Stability Variation for Human Health

While we know that genetically encoded variation in microtubule stability affects pyroptosis, we suspect that the same genetic difference that alters *TUBB6* expression would affect any cellular trait that depends on microtubules. There are two limitations to this statement. First, the association is with a common SNP in healthy individuals, so the effect must be a quantitative difference compatible with a relatively healthy life. Second, there are likely threshold effects with microtubule stability where the degree of variation we observe due to *TUBB6* has no effect. For example, we found that while paclitaxel treatment to stabilize microtubules in THP-1 monocytes increased pyroptosis in response to *Salmonella*, it had no effect when the cell line was differentiated into macrophage-like cells. The apparent reason for this is that the differentiation program from monocytes to macrophages results in greater microtubule stability (that we suspect is due to downregulation of *TUBB6* expression, which we observed). Thus there may be cell types and specific cellular processes that are more or less susceptible to genetically encoded inter-individual variation in microtubule stability.

Two areas that could be impacted by our finding are cancer therapy and

neurobiology. Paclitaxel has long been used in the treatment regimens of multiple cancers, and we suspected that sensitivity or toxicity to this drug might be affected by the *TUBB6* SNP. While mining the data in a previously published study in LCLs²³ found no association between cell death induced by paclitaxel in the LCLs and the *TUBB6* SNP, we suspect that effects could be being masked by variation in drug transporter expression. It is still worth examining in patients undergoing cancer treatment where paclitaxel can cause sensory neuropathy, especially at higher doses.²⁴

Numerous rare mutations in β and α tubulin isoforms have been associated with various severe neurological disorders.^{25,26} Several de novo missense mutations in *TUBB2B* (tubulin, β 2B class IIb) and *TUBA1A* (tubulin, α Ia) have been associated with a spectrum of lissencephalic and polymicrogyric cortical dysplasias.²⁷⁻²⁹ Mutations in *TUBB3* cause a range of malformations of cortical development and congenital fibrosis of the extraocular muscle type 3.^{30,31} These mutations are predicted to act by disrupting β/α tubulin dimerization, microtubule polymerization, or the binding of microtubule-associated proteins. These disruptions of protein-protein interactions result in drastic alterations in neuronal and axonal differentiation and transport.^{28,30,31} Could common variants that alter the expression of *TUBB6* or other tubulin isoforms have more subtle effects on neuronal differentiation, transport, and function? Could common neurological disorders such as Alzheimer disease be impacted by this common variation in microtubule stability? These unanswered questions warrant further study. The *TUBB6* SNP has not emerged out of any GWAS of patient populations yet, but the vast majority have focused on risk of disease, and variation in *TUBB6* expression might have an effect instead on severity.

On to More Studies of Natural, Common Variation in Cell Biological Traits

Our cellular GWAS studies of pyroptosis demonstrate that GWAS of cellular

traits can lead to interesting insight into cell biological processes involved in host-pathogen interactions. More generally, since pathogens are master manipulators of normal cellular physiology, the findings from a particular response to a specific pathogen are truly more broadly applicable. *Salmonella* pyroptosis is a readout for cell variation in caspase-1 signaling and (as the *TUBB6* variant would suggest) microtubule and plasma membrane stability. Additional screens, for example, on *Salmonella* invasion serve as more general screens for macropinocytosis regulated by Rac1 and phosphoinositide metabolism. As we continue to add more pathogens and treatments to the list of cellular GWAS studies, the end result will be a catalog of how human genetic variation affects cell biological traits. This will not only lead to new discoveries in cell biology but a resource to interpret and provide experimentally tractable systems to study the catalog of genetic variants associated with human disease.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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